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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

My long-term goal is to identify new components of tumor suppressor networks that are important in breast cancer. My initial proposal focused on apoptosis, and how components of the 'apoptotic machinery' influence breast carcinogenesis and chemotherapeutic responses. For several reasons, these studies were abandoned to focus on cellular senescence. Senescence is a permanent cell cycle arrest program that is conceptually similar to apoptosis and is controlled by the p53, p16, and Rb tumor suppressors (which are important in breast carcinogenesis). I am currently identifying components of the 'senescence machinery' and determining how they suppress proliferation. I have shown that senescence is accompanied by changes in chromatin structure that depend on the p16/Rb tumor suppressor pathway and lead to the repression of growth regulatory genes. I have also generated chimeric mice harboring ES cells with a targeted disruption of one putative component of the silencing program. Future studies will examine the impact of these mechanisms on transformation in vitro and tumorigenesis in vivo, as well as their ability to modulate the cytotoxicity of anticancer drugs. These studies are guided by our understanding of apoptosis, and promise to provide new insights into a new program of tumor suppression in breast cancer.

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Introduction

Our laboratory has long been interested in the process of apoptosis, a process of programmed cell death that can be induced in response to aberrant proliferative signals and many anticancer agents. As a consequence, disruption of apoptosis can lead to tumor progression and drug resistance. In our original application, we proposed to study how apoptosis is executed and the implications of disrupting components of the 'apoptotic machinery' on breast carcinogenesis. Interestingly, senescence is an anti-proliferative program that has many parallels to apoptosis. First, senescence is controlled by important tumor suppressors, including ARF, p53, p16, and Rb, and acts to permanently limit cell proliferation. Second, various stimuli, including oncogenic ras and DNA damage, can induce senescent phenotype, suggesting the existence of a common senescence machinery that contributes to the irreversible nature of the arrest (1-3). Third, senescence functions as an important brake to tumor development and contributes to the action of certain cytotoxic anticancer agents. Together, these observations suggest that senescence parallels apoptosis as an antitumor cellular response to stress. We hypothesize that this process is important in breast cancer development and treatment responses.

As indicated above, senescence appears to involve the Rb and p53 tumor suppressor pathways. Hence, as cells enter a senescent state, p53 and p16 accumulate, and Rb becomes hypophosphorylated. In breast cancer, approximately 50 % of tumors show low or lack of p16 expression, and many have mutations in Rb or p53. It is also significant that cells derived from mammary tissues almost always lose p16 expression when cultured in vitro (4). These observations may be indicating a higher propensity of mammary cells compared to other cell types to inactivate the senescence pathway by loss of p16.

For several reasons (see proposal body), we have abandoned our initial aims to produce mice with defects the p53 effectors Caspase-9 and Apaf-1 to identify and characterize components of the senescence machinery. Specifically, we are interested in how cells initiate and maintain the senescent state, and the role of the p53 and p16/Rb pathways in the process. Based on the established involvement of p53, p16, and Rb in breast carcinogenesis, we hypothesize that cellular senescence is an important mechanisms of tumor suppression that is disrupted during breast cancer development. Moreover, our laboratory has recently shown that a senescence program controlled by p53 and p16 contributes to the outcome of cancer therapy in tumors, and so insights into how

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senescence is initiated and maintained may have important implications for understanding drug sensitivity and resistance in breast cancer, and may ultimately help identify better cancer therapies.

Although these experiments differ substantially from our initial proposal, they are conceptually similar in that they are (i) guided by parallels between senescence to apoptosis; (ii) focused on the terminal stage of this anti-proliferative program; (iii) directed towards uncovering new insights into molecular mechanisms of tumor suppression and drug action. Our progress has been substantial, and we are currently writing up the first study that we hope will have a large impact on the field. Although details of our results will be discussed in the 'Body of the Annual Report', it is noteworthy that we have implicated the HP1 proteins in the maintenance phase of senescence. HP1α is down regulated in a substantial proportion of breast cancers (5), which provides additional links between our studies and breast carcinogenesis. In short, we have based our transition from work on apoptosis to senescence on exciting results, and believe our work is as relevant to breast cancer (if not more) that the studies in our initial proposal.

Body of Annual Report

The ultimate goal of our project is to find uncover the molecular mechanisms of tumor suppression in breast cancer, which include apoptosis and cellular senescence mechanisms. The objectives outlined in my research proposal were designed to elucidate the mechanism of the tumor suppression through the p53-dependent apoptosis pathway, using a genetic approach. Specifically, we proposed to generate conditional knockout mice of Apaf-1, which is downstream target of p53 pathway (6). However, we found that at least 2 groups already generated these mice, putting us at a serious disadvantage. In addition, we obtained some extremely exciting results on the area of cellular senescence. Therefore, we shifted the theme from apoptosis to cellular senescence.

Senescence was initially identified as a permanent form of cell cycle arrest that accompanied the replicative exhaustion of human fibroblasts in culture. However, recent work from our laboratory and elsewhere has shown that senescence is induced by many forms of cellular stress. By analogy to apoptosis, we have been seeking the "common machinery" of cellular senescence. A large body of evidence indicates that both the p53 and Rb tumor suppressor pathways contribute to cellular senescence. Our laboratory and others reported that p53 is required for the induction of 'premature' senescence by oncogenic ras (1, 2, 7). However, p53 is not essential for maintaining senescence (8). On the other hand, there are several lines of observation, which suggests the involvement of epigenetic regulation of gene expression upon senescence (9). Furthermore, we recently found that senescent human fibroblasts exhibit the condensed chromatin structure, which shares heterochromatic features, and that the formation of these structures was strictly dependent on the presence of an intact p16/Rb pathway (Narita et al., in prep). Based on these findings, we hypothesize that epigenetic determination of senescence-specific gene expression pattern may be important for maintaining the stable growth arrest in senescence. Here we focus on our findings so far about cellular senescence and further experimental plans.

Characterization of chromatin structure in senescent HDFs

IMR90s, human diploid fibroblasts (HDFs), induced to senesce by oncogenic Ras, as well as replicative senescent fibroblasts, show a characteristic DAPI staining pattern, exhibiting condensed DAPI-dense foci interspersed throughout the nucleus (Figure 1A). We have designated this as SAHR, Senescence Associated Heterochromatic Regions, because it has heterochromatic features as shown below. First we examined the relationship between SAHRs formation and senescent

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phenotype. We have found a close correlation between SAHRs formation and senescent markers such as, cellular morphology and senescence associated β-galactosidase (SA-β-gal) activity (Figure 1B). Furthermore, the adenoviral oncoprotein E1A, which overrides senescence, also inhibits SAHRs formation completely. SAHRs are not found in quiescent cells, indicating SAHRs are not consequence of cell cycle arrest. Taken together, these results indicate that SAHRs could be a new marker of senescence.

To characterize SAHRs, we examined the components of SAHRs, and found that SAHRs are heterochtomatic structure. First, HP1 proteins, a family of heterochromatic adaptor molecules involved in epigenetic gene regulation and supra-nucleosomal chromatin structure, colocalized with SAHRs by immunostaining (Figure 2), suggesting that HP1 proteins are involved in SAHRs-formation upon senescence. Interestingly, HP1α is downregulated in a substantial proportion of breast cancers (5). Next, acetylated histone H3 (K9/14), which is consistent with euchromatin, was clearly excluded from SAHRs (Figure 3). Third, SAHRs colocalized with histone H3 Lys9 methylation (K9M), which provides a binding site for HP1 proteins contributing to proper assembly of heterochromatin (10-12), but did not colocalize with H3 Lys4 methylation (K4M), which was shown to be associated with active chromatin (13, 14) (Figure 3). Histone modification pattern of SAHRs shown here is highly suggestive of transcriptinally inactive chromatin structure.

Heterochromatic features of SAHRs were further confirmed by electron microscopy. Similar to DAPI staining, we identified the well-demarcated regions with relatively high electron density, which is consistent with heterochromatin, in the nuclei of ras-senescent cells, but not in vector control or low serum quiescent cells (Figure 4A-C). Immuno-gold labeling with anti-DNA antibody showed DNA-enrichment in the SAHRs (Figure 4D). Consistently, RNase-gold labeling revealed that RNA was absent from the SAHRs (Figure 4E).

Global level of acetylation of histone H3 was decreased in ras-senescent as well as replicative senescent cells by Western blotting (Figure 5A), while quiescent cells did not show any alterations in the level of H3 K9/14Ac (Figure 5A). E1A again prevented hypo-acetylation of H3 K9/14 in response to oncogenic ras. Consistently, chromatin bound fractions of HP1β and HP1γ were increased in ras-senescent cells compared to vector control and quiescent cells (Figure 5B). E1A abolished the accumulation of HP1 proteins in chromatin fractions in response to oncogenic ras (Figure 5B). Together, these results indicate that oncogenic ras, as well as replicative exhaustion induced distinct chromatin structure, which is a novel type of heterochromatic structure. Furthermore, decrease in global level of H3 acetylation and increase in

chromatin bound HP1s in senescent cells suggest that eu- and heterochromatin redistribution may associate with the process of senescence in human fibroblasts.

p16^{INK4a}, which expression is shown to be low or missing in many human cancer cells, including breast cancer, accumulates in senescent cells, and also can induce some features of cellulare senescence (4, 14, 15). We asked if p16^{INK4a} also induces SAHRs in IMR90s. p16^{INK4a} was introduced into IMR90s by retroviral-mediated gene transfer, followed by SA-β-gal assay, BrdU incorporation assay, and DAPI staining. Consistent with previous reports, p16^{INK4a} induced the senescence phenotype, including SA-β-gal activity and low DNA synthesis (Figure 6A). p16^{INK4a} also induced SAHRs, which were indistinguishable from the nuclei of ras- or replicative senescent cells (Figure 6B).

Regulation of SAHR formation and impact on senescent state

The data described above demonstrate that alterations in chromatin structure accompany senescence. Recent work has suggested that these changes lead to the repression and/or silencing of growth regulated genes (not shown). Moreover, the adenovirus E1A oncoprotein can interfere with the induction of these structures in a manner that depends in its ability to inactivate Rb, implying that an intact Rb pathway is required for the process. We are currently testing this hypothesis directly using novel 'short-hairpin' RNAs. To this end, we are collaborating with Dr. Greg Hannon (CSHL) to produce retroviral vectors capable of stably suppressing gene expression in fibroblasts and breast epithelial cells. Our preliminary results suggest that this approach will be successful at completely suppressing the expression of genes such as Rb and p16.

Generation of HP1 knockout mice

The putative role of HP1 proteins in maintaining senescence is consistent with a tumor suppressor activity and, consistent with this view, HP1α is not expressed in a subset of breast cancers (5). In collaboration with Dr. Harald Von Melchner (University of Frankfurt), we have obtained ES cells that that have insertional mutations in the HP1α and HP1β genes. The HP1α+/- ES cells have produced chimeric mice and the HP1β+/- cells have gone germline. We will use these cells to study the role of HP1 in normal development and in cancer.

Future plans

1. Molecular mechanism of SAHR formation

To dissect the Rb/p16 and p53 pathway in the SAHRs formation, we will make the retroviral short

hairpin RNAs (shRNA) to obtain the stable silencing. Candidate genes to be silenced include Rb, p16, p53, and HP1s. We will analyze the correlation between chromatin structure and phenotype, or gene expression pattern.

2. Chromatin status in the specific genes

To obtain the direct evidence for the epigenetic regulation of gene expression, chromatin immunoprecipitation (ChIP) analysis using antibody against Lys9/14 acetylated histone H3 (euchromatic marker), or Lys9 methylated H3 (heterochromatic marker) will be performed. We will focus on the E2F-target genes, such as cyclin A and PCNA, which are essential for cellular proliferation, and marker genes for senescence, such as stromelysin 1.

3. Analysis of mammary epithelial cells.

We will examine epithelial cells for SAHRs and senescence-associated epigenetic changes in gene expression to determine the similarities and differences with fibroblasts. If substantial differences are observed, we will focus on mammary epithelial cells owing to their increased relevance to breast cancer.

4. In vivo models

The association between down regulation of HP1α expression and metastatic phenotype of breast cancer was recently reported. In parallel to our initial proposal on Apaf-1, we will generate mice with targeted disruptions in HP1α and HP1β (and HP1γ if possible). These animals will be studied for spontaneous cancer formation, and crossed to standard models of breast carcinogenesis (e.g. MMTV-ras).

Based on this Future Plan, new "Statement of Work" will be submitted separately.

Key Accomplishments

- Ras induced characteristic chromatin structure, SAHRs, which is indistinguishable from replicative senescent cells in IMR90s.
- SAHRs show close correlation with senescent phenotype.
- SAHRs show heterochromatic features.
- E1A prevents SAHRs formation by ras in IMR90s.
- p16^{INK4a} also induced SAHRs.

Reportable Outcomes

Poster and Abstact

Masashi Narita, Edith Heard, Stephen Hearn, Masako Narita, Scott Lowe; *SAHR, a new marker of senescence, which exhibit heterochromatic features* (poster). 11th International p53 Workshop, Barcelona, Spain, May 15-19, 2002

Masashi Narita, Edith Heard, Stephen Hearn, Masako Narita, Scott Lowe; *Higher order chromatin structure and cellular senescence* (poster). Cancer Genetics and Tumor Suppressor Genes, CSHL, August 14-18, 2002

Conclusion

We have found the novel type of heterochromatic structure (SAHRs) associated with senescence phenotype in human fibroblasts.   Rearrangement of chromatin structure is strongly suggestive of the involvement of epigenetic regulation in senescence.   One candidate pathway for SAHRs formation is the p16/Rb pathway, at least in part, because E1A blocks the SAHRs and p16 is sufficient to induce SAHRs.   Other components of the process involve the HP1 proteins, which may ultimately lead to the silencing of growth related genes.   Understanding the molecular mechanism of SAHRs formation and the epigenetic regulation of the specific genes expression in senescence are on ongoing and could contribute to elucidating the "common pathway" of cellular senescence as the machinary of tumor suppression.

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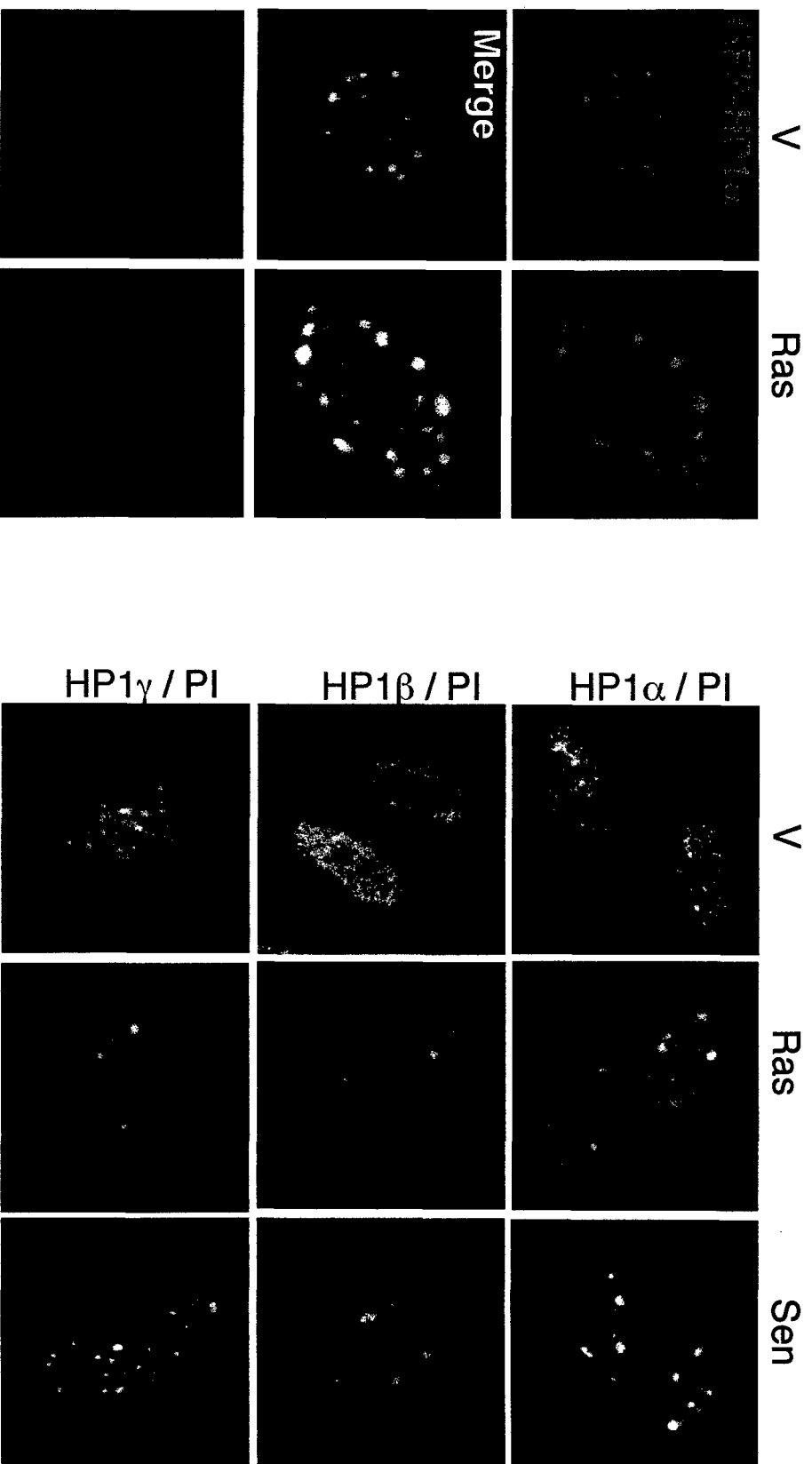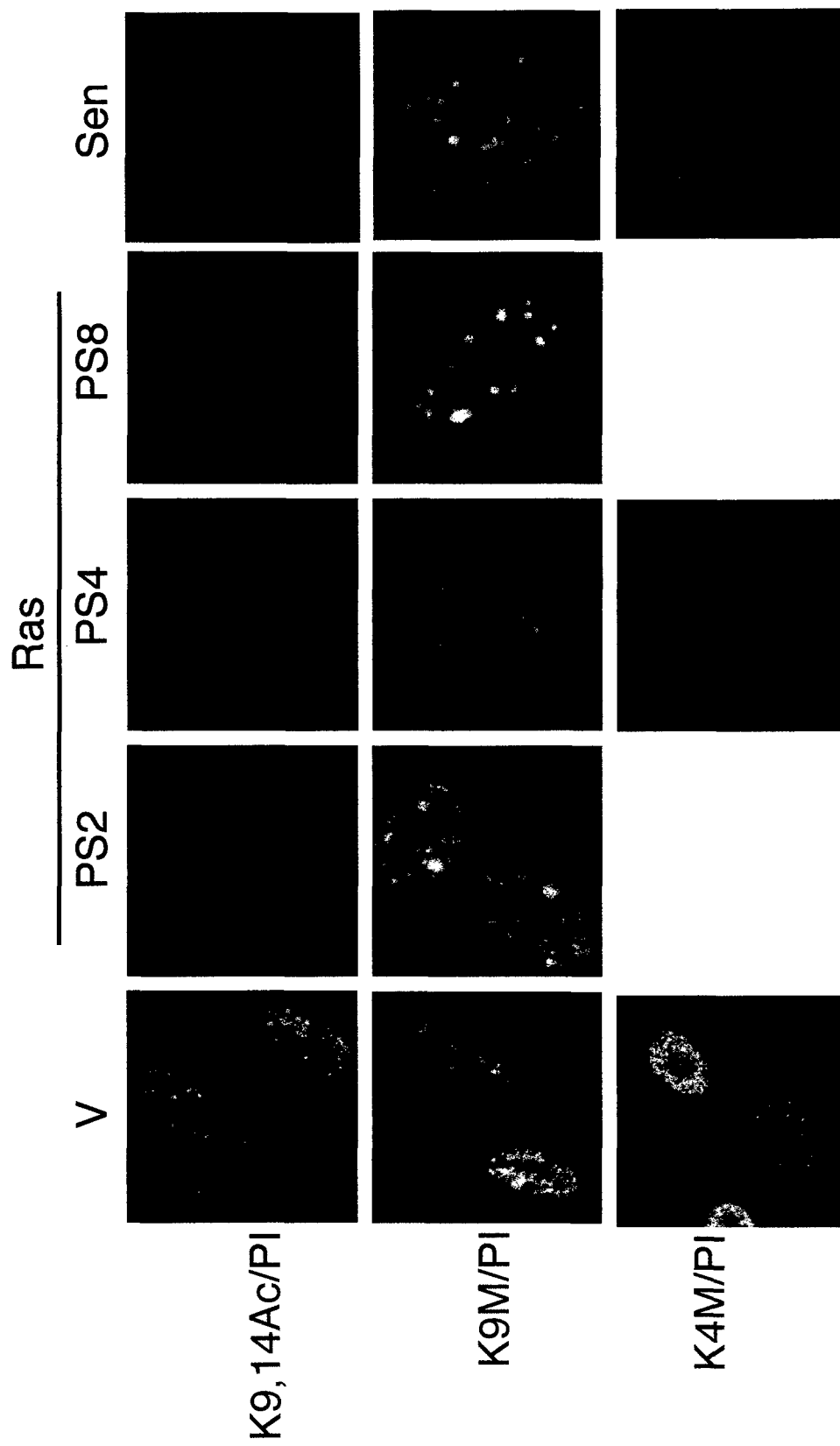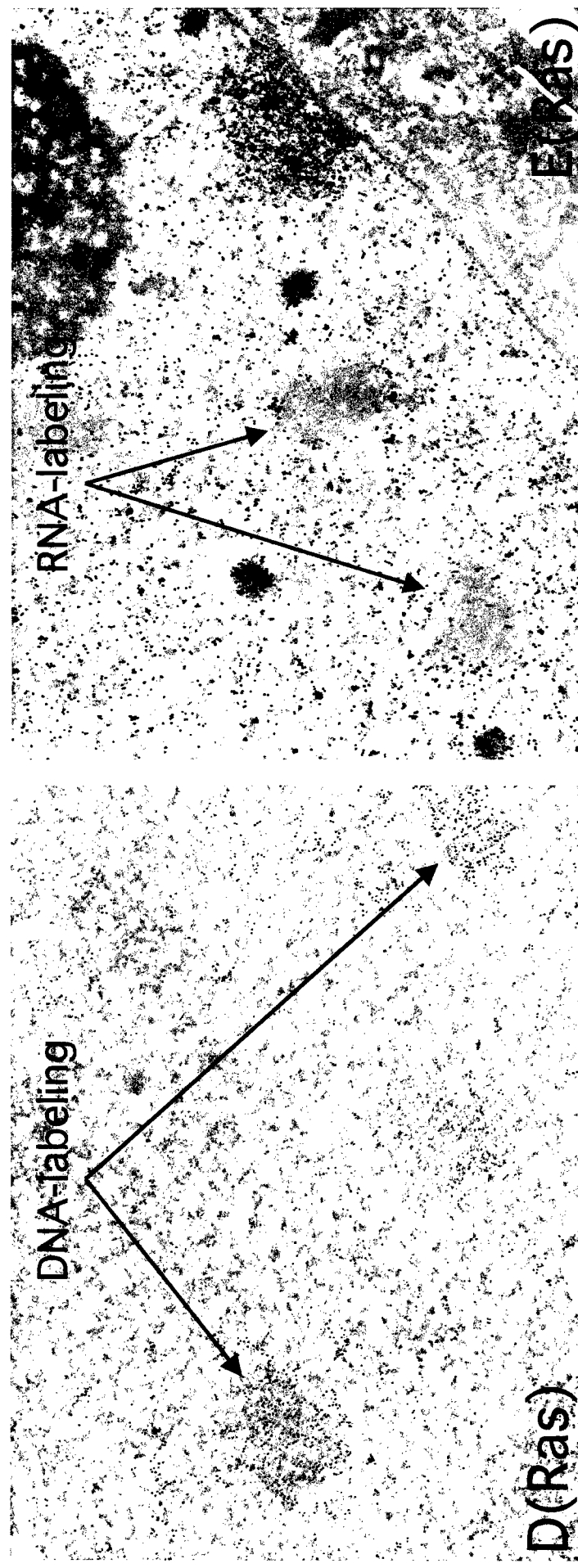
Figure 1

Figure 2

Figure 3

Figure 4

A(Ras) Nucleolus

B(V)

C(LS)
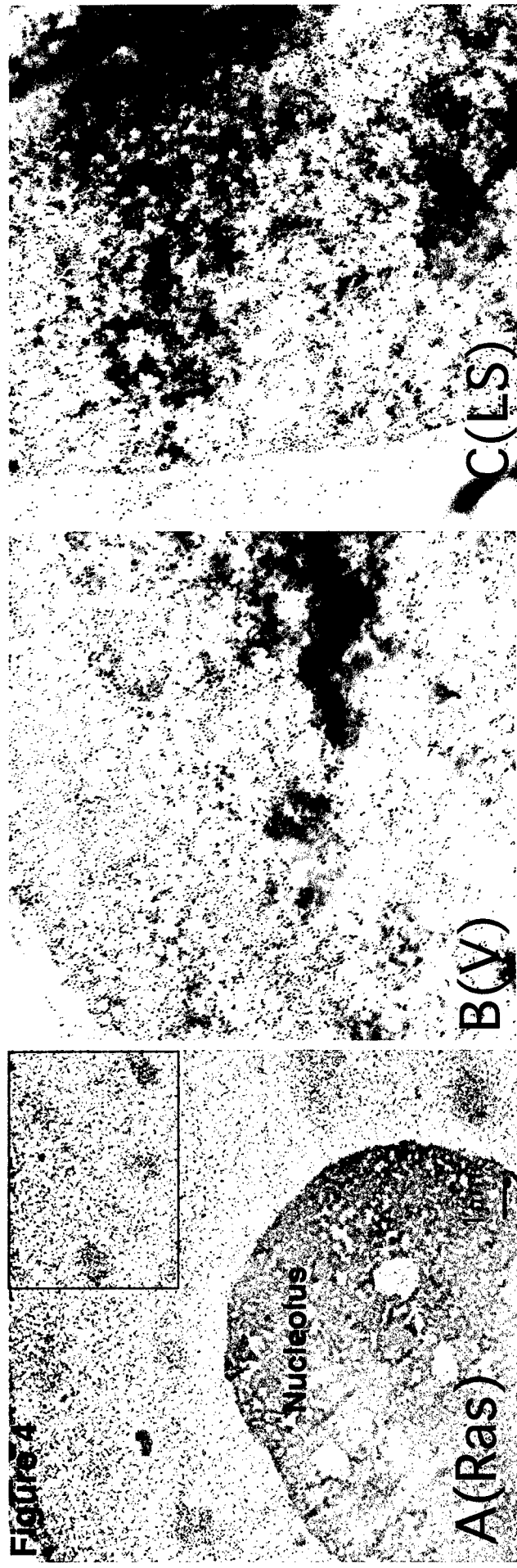
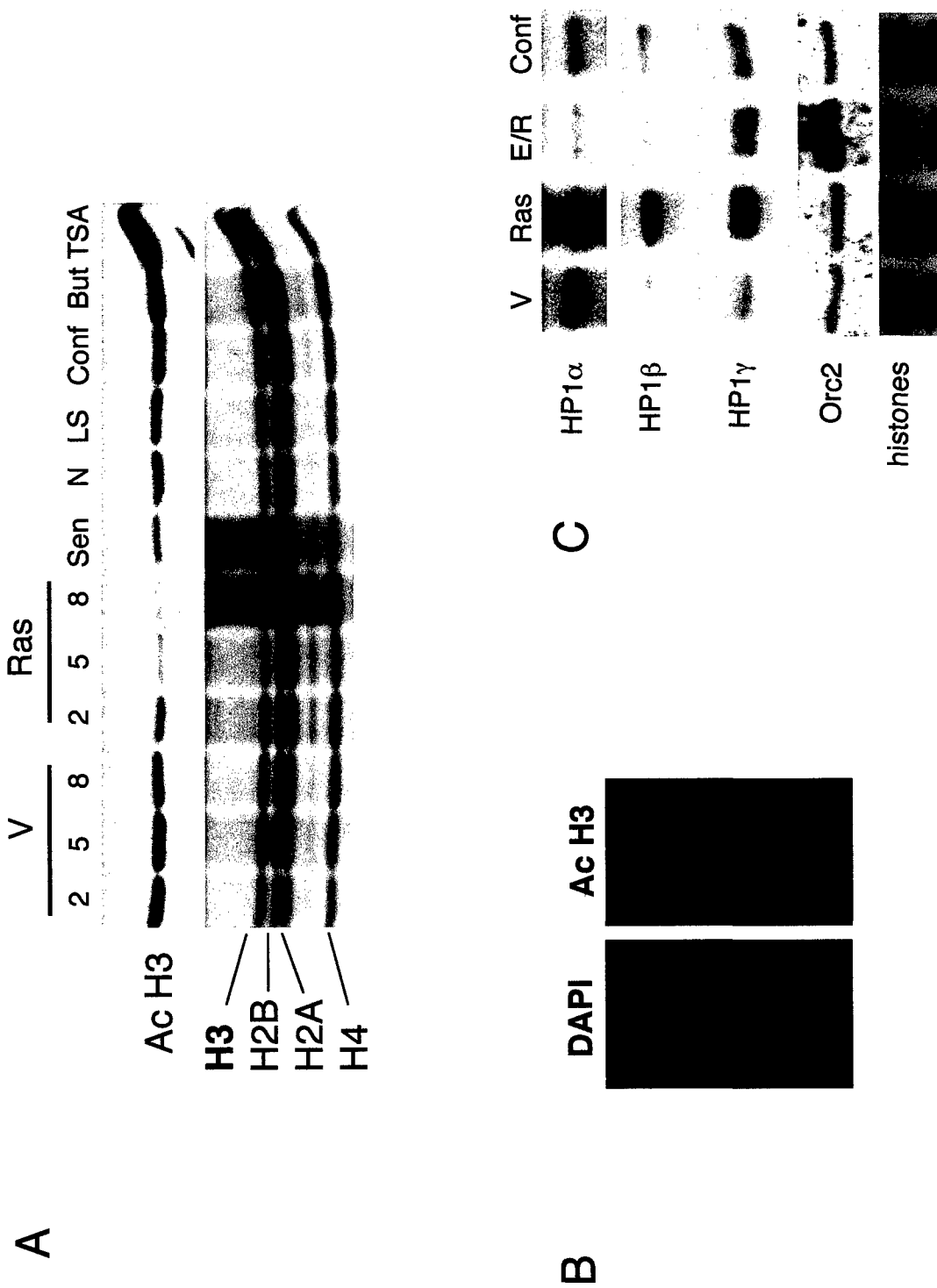D(Ras) DNA-labeling

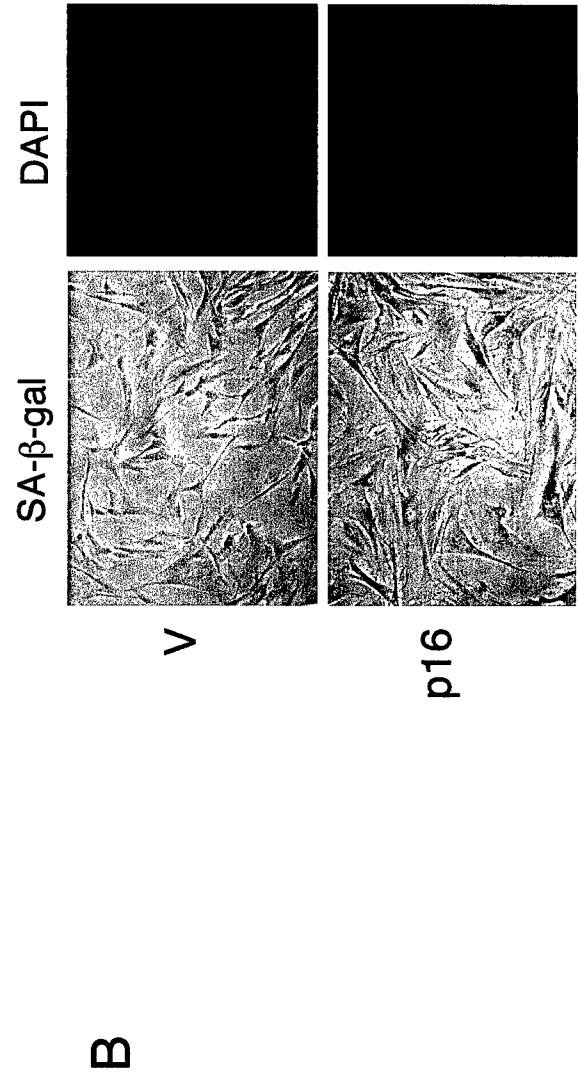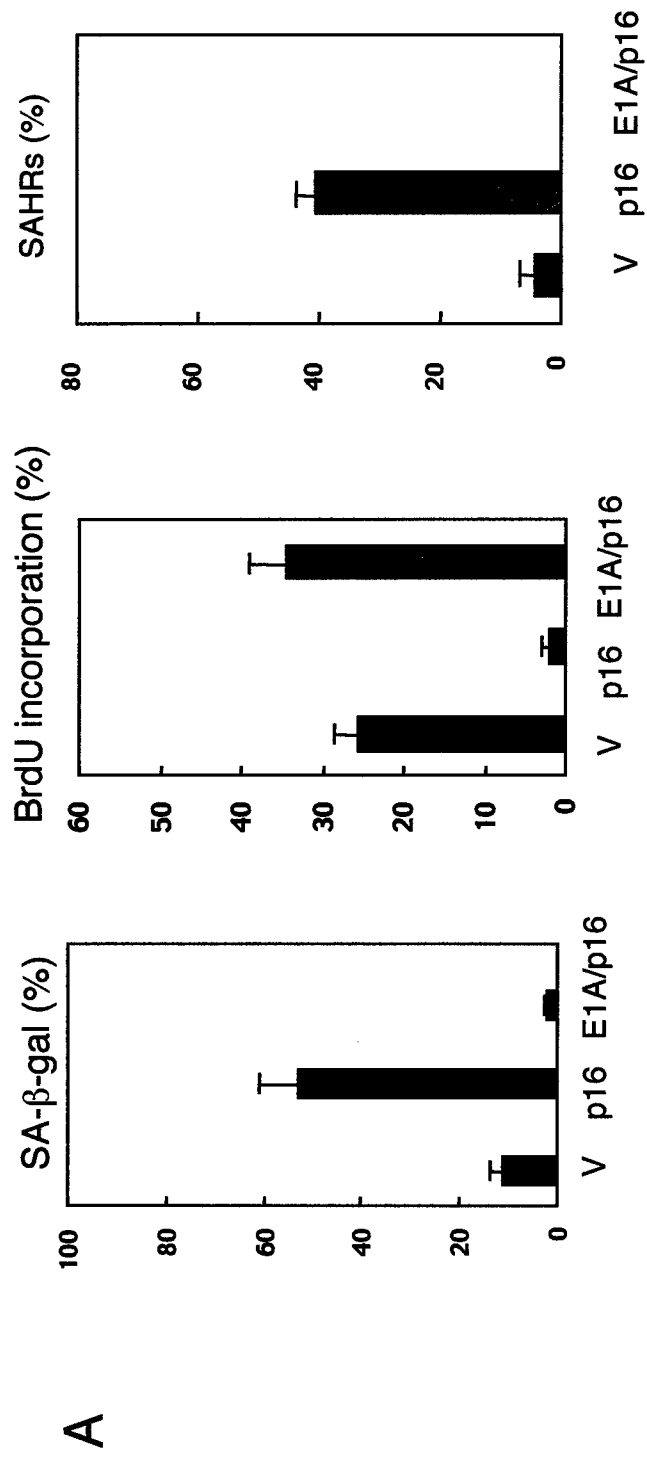E(Ras) RNA-labeling
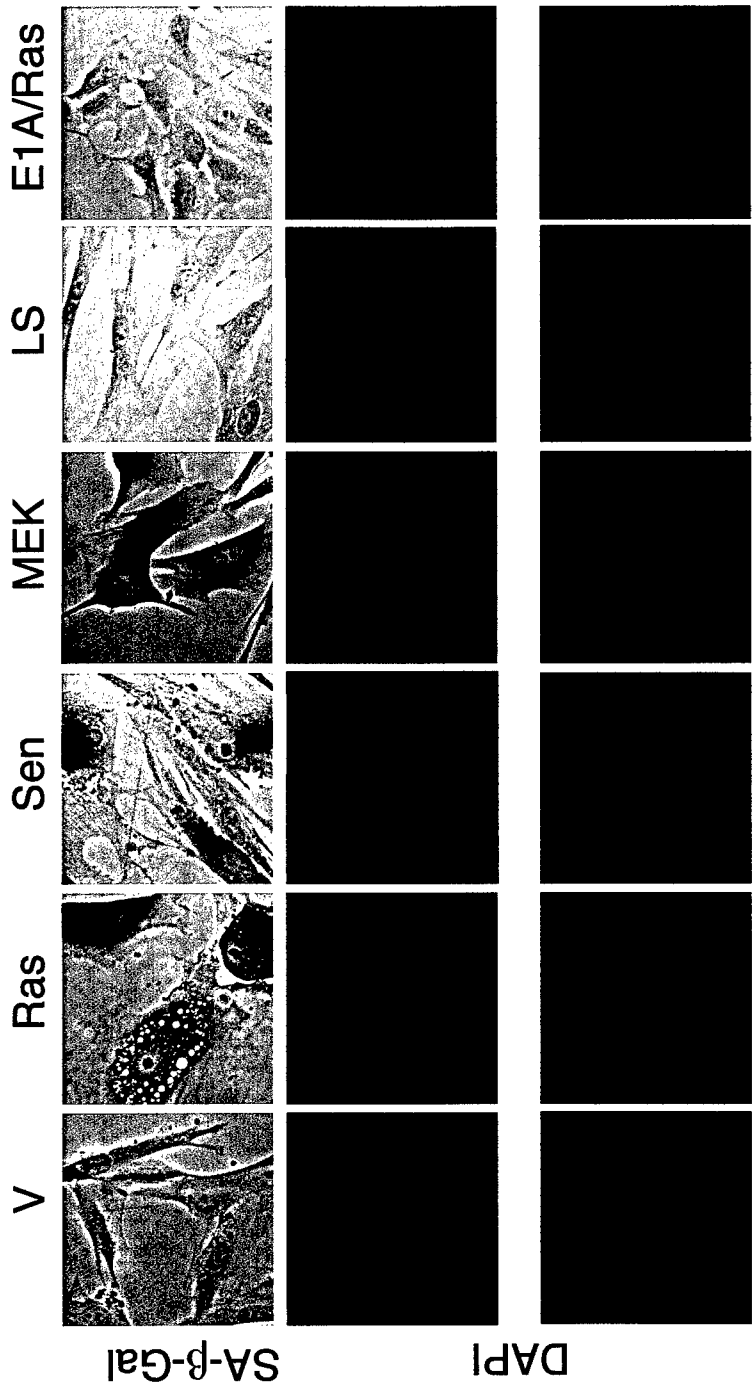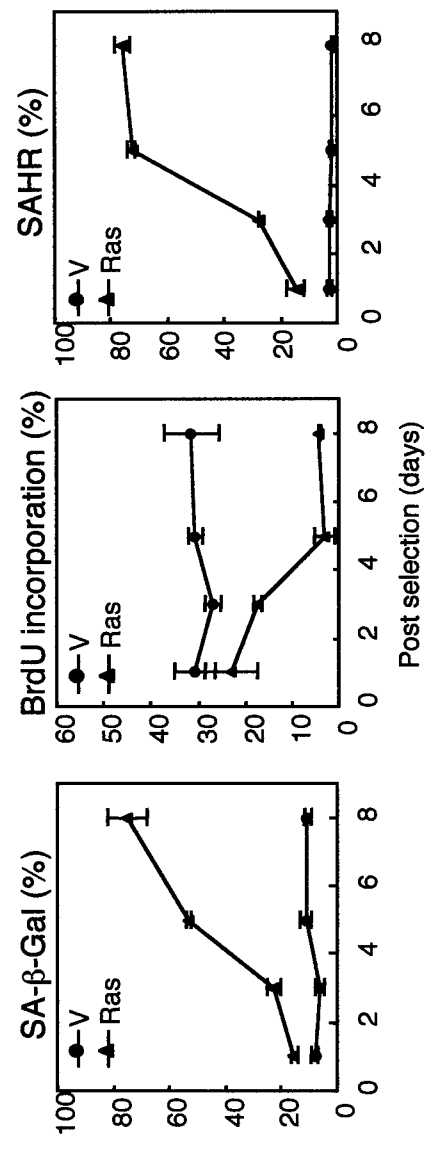
Figure 5

Figure 6

Figure Legends

Figure 1. Senescence associated nuclear mórphology in IMR90s

(A) IMR90s containing empty vector (V), H-RasV12 (Ras), or E1A12S/H-RasV12 (E/R) were stained for SA-b-galactosidae activity, followed by DAPI staining, at day 6 postselection. Late passage IMR90s (Sen) and quiescent cells prepared by low serum (LS) were also shown for comparison. Enlarged images of DAPI staining were shown in the lower panels.

(B) Percentage of SA-β-gal (left panel) and SAHR (right panel) positive cells at indicated days after selection was scored in IMR90s containing empty vector (V) or H-RasV12 (R). DNA synthesis was monitored by BrdU incorporation (middle panel) at the same time course.

Figure 2. HP1 proteins colocalize with SAHR

Colocalizatioin of GFP-fused HP1α or endogenous HP1s (HP1α, β, and γ) and SAHR by laser scanning confocal microscopy. GFP-fused HP1α was co-expressed with either empty vector (V) or H-RasV12 (Ras) by retroviral gene transfer. Localization of endogenous HP1s was determined by indirect immunofluorescence using the respective antibodies in V, Ras, and replicative senescent cells (Sen). DNA was stained with propidium iodide (PI).

Figure 3. Modification of histone H3 in senescent IMR90s

Confocal images of histone H3 Lysine 9/14 acetylation (K9/14Ac), Lysine 9 methylation (K9M), and Lysine 4 methylation (K4M) by indirect immunofluorescene using rabbit polyclonal antibodies, in IMR90s expressing empty vector (V) or H-RasV12 (Ras), as well as replicative senescent cells. DNA was counter-stained by propidium iodide (PI).

Figure 4. DNA/RNA labeling in senescent nuclei of IMR90s

(A-C) Electron microscopy showed the similar condensed chromatin pattern as DAPI staining in ras-senescent cells (Ras), but not in control vector (V) and low serum (LS) quiescent cells. DNA was detected with monoclonal anti-DNA antibody using gold-coupled secondary antibody. **(D)** Higher magnification view of SAHR in **A** was shown. **(E)** RNA was labeled with gold-coupled RNase T1 in the same magnification as **D**.

Figure 5. Global level of H3 acetylation or chromatin-bound HP1s

(A) Downregulation of acetylated histone H3 in senescent cells. Equivalent amounts of chromatin-enriched fraction (normalized by cell number) prepared from indicated cells were

subjected to SDS-PAGE for acetylated H3 immunoblotting or Coomassie blue (CBB) staining. As positive controls, young IMR90s were treated with HDAC-inhibitors, sodium butyrate (But; 5mM, 24h), or trichostatin A (TSA; 100ng/ml, 4h). Normal young IMR90s (N), low serum (LS), and confluent cells (conf) were shown for comparison. **(B)** Representative immunofluorescent image indicating different level of H3 acetylation between nuclei with and without SAHR. **(C)** Increased level of chromatin-bound HP1β/γ in Ras-senescent cells. Chromatin-enriched fraction prepared from the equal number of cells were subjected to Western blotting of HP1α, β, and γ. Orc2 Western blot and CBB staining served as loading controls.

Figure 6. p16 induces SAHRs in IMR90s

(A, B) IMR90s containing empty vector (V), p16 (p16), and both E1A and p16 (E1A/p16) were assessed for SA-b-galactosidae activity, DAPI staining, and DNA synthesis at day 4 postselection, as in Figure 1.

Abstract

11th International p53 Workshop,

Title: SAHR, a new marker of senescence, which exhibit heterochromatic features

Masashi Narita, Edith Heard, Stephen Hearn, Masako Narita, Scott Lowe.
Cold Spring Harbor Laboratory

Human diploid fibroblasts (HDFs), such as IMR90s, enter an irreversible growth arrest after a limited number of divisions in culture, known as replicative senescence. Oncogenic ras induces premature senescence, which is phenotypically indistinguishable from replicative senescence. Cellular senescence is accompanied by the accumulation of the tumor suppressors p53 and p16. Both the p53 and p16/Rb pathways must be circumvented for HDFs to escape from senescence.

While characteristic morphological changes in senescent human fibroblasts are well known, the nuclear morphology of senescent cells is poorly characterized. Ras-induced senescent IMR90s, as well as replicative senescent fibroblasts, show a characteristic DAPI staining pattern, which we have designated as SAHR, Senescence Associated Heterochromatic Regions. We have found a close correlation between SAHR formation and senescnece markers such as, accumulation of p53/p16 and senescence-associated β-galactosidase activity. Furthermore, the adenoviral oncoprotein E1A, which overrides senescence, also inhibits SAHR formation, and SAHRs are not found in quiescent cells. Taken together, these results indicate that SAHRs are a marker of senescence.

Immunofluorescence studies reveal that SAHRs colocalize with markers of heterochromatin, such as HP1 (heterochromatin protein 1) and Lysine 9 methyled histone H3. In contrast, lysine 9/14 acetylation and lysine 4 methylation of histone H3, which are consistent with euchromatin, are excluded from SAHRS. Electronmicrography confirms similar heterochromatic structures in senescent cells, where DNA is condensed and RNA is absent. Colocalization studies have excluded regions of constitutive heterochromatin, centromere and telomere, as being part of the SAHR, suggesting that SAHRs are a novel type of heterochromatic structure. Given the fact that the levels of acetylated histone H3 are downregulated in senescent cells, our data suggest a global rearrangement of chromatin structure during senescence. If this is true, it could provide a specific gene expression pattern particular to senescence.

Abstract

HIGHER ORDER CHROMATIN STRUCTURE AND CELLULAR SENESCENCE

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June/1994- May/1995   Medical Staff in Department of Surgery, Osaka Police Hospital

June/1995- May/1996   Clinical Fellow, Kinki-Chuo National Hospital

April/2000- pres.   Postdoctoral Research, Cold Spring Harbor Laboratory

PERSONAL INFORMATION

Date of Birth: June 19, 1963

Place of Birth: Kyoto, Japan

Nationality: Japanese

Marital Status: Married, 1993

LICENSE AND CERTIFICATION

1990 FMGEMS Basic Component

1991 FMGEMS Clinical Component

1992 Japanese Medical License Registration

Memberships

The Molecular Biology Society of Japan

Japanese Cancer Association

Japan Surgical Society

MAJOR RESEARCH INTERESTS

1. apoptosis

2.oncology

Honors and Awards:

Apr/2000   Uehara Memorial Foundation (Japan), Research fellowship

Jul/2001   Department of Defense, Breast Cancer Research Program

                     Postdoctoral Fellowship Award

PUBLICATIONS

Original articles

1. Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc Natl Acad Sci USA* 1998 Dec 8; 95(25): 14681-6

2. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 1999 June 3; 399: 483-7

3. Nomura M, Shimizu S, Ito T, Narita M, Matsuda H, Tsujimoto Y. Apoptotic cytosol facilitates Bax translocation to mitochondria that involves cytosolic factor regulated by Bcl-2. *Cancer Res.* 1999 Nov 1; 59(21): 5542-8.

4. Yamabe K, Shimizu S, Ito T, Yoshioka Y, Nomura M, Narita M,, Saito I, Kanegae Y, Matsuda H. Cancer gene therapy using a pro-apoptotic gene, caspase-3. *Gene Ther.* 1999; 6: 1952-9.

5. Satokata I; Tanaka K; Miura N; Narita M; Minaki T; Satoh Y; Kondo S; Okada Y. Three nonsense mutations responsible for group A xeroderma pigmentation. *Mutat Res* 1992 Mar; 278(2): 193-202.

6. Narita M, Nakao K, Ogino N, Nakahara M, Onishi A, Tsujimoto M. Independent prognostic factors in breast cancer patients. *Am J Surg* 1998 Jan; 175(1): 73-5.

7. Narita M, Nishida T, Nakao K, Ogino N et al. Prognostic factors in breast cancer and their limitations. SURGICAL TECHNOLOGY INTERNATIONAL VIII 1999: 289-94 (review).

8. Narita M, Nakao K, Ogino N, Emoto T, Nakahara M, Yumiba T, Tsujimoto M. A case of Microangiopathic Hemolytic Anemia Associated with Breast Cancer: Improvement with Chemoendocrine Therapy. *Breast Cancer* 1997; 4:39-42.